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Methods for the Isolation and Identification of Steroidal Sapogenins

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During the period January - May 1950, methods for the isolation and identification of steroidal sapogenins have been intensively studied. Old and new techniques have been combined to give procedures applicable to a large number and variety of plant samples. The attached flowsheet outlines the various steps in the procedure. Brief descriptions of the steps and a typical analysis are presented in the following text.

Raw Material Processing

Steps 1-4 involve processing fresh plant samples to a dry meal and are self explanatory. The meal obtained in step 4 must be ground rather coarsely in order to avoid mechanical difficulties during the alcohol extraction.

Extraction

Sapogenins occur in a glucosidic combination called saponins. Steps 5-7 involve preparation and "de-fatting" of an alcoholic saponin extract. The meal is stirred for one hour with 70% ethanol at reflux temperature. The ethanol is filtered, and the residual meal is then exhaustively extracted with 95% ethanol in large Soxhlet extractors. The alcoholic extracts are combined, concentrated to a volume of 1-2 liters and the alcohol concentration roughly adjusted to 50% alcohol by volume. The resultant aqueous ethanol extract is placed in a column packed with Raschig rings and continuously extracted with benzene for 4-5 hours. The benzene extraction removes fats, sterols, and pigments which could later cause trouble.

Hydrolysis

The sapogenins are isolated and purified by the methods outlined in steps 8-16. Sufficient concentrated HCl is added to the aqueous alcohol extract from step 7 to make the final concentration 2N. The extract is placed in the packed column used previously, the temperature raised to 65°C., and the extract continuously extracted with benzene for 5-6 hours. Under these conditions, the saponins are slowly hydrolyzed and the benzene soluble sapogenins are removed as fast as they are released. In this way changes in sapogenin structure due to prolonged exposure to hot HCl are avoided.

The sapogenin extract at this stage (step 8) is quite impure, containing only 1-10% sapogenins. Alkali treatment (step 10) removes phenolic compounds and resin acids, raising the sapogenin purity to 10-20%.

Molecular Distillation

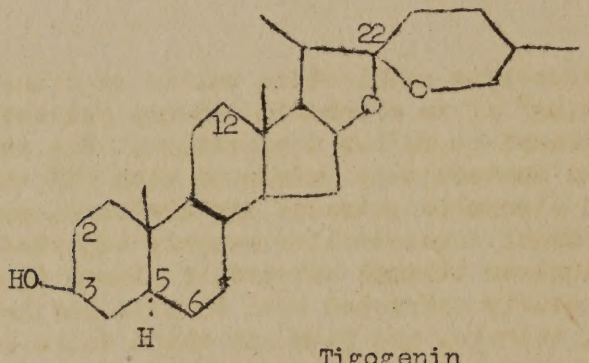
Molecular distillation or sublimation markedly raises the sapogenin purity to 25-75%. Preliminary tests with known sapogenins indicated that the process would give quantitative yields with no alteration in the sapogenins tested. The procedure is applied to the benzene extract from step 11 as follows: The benzene is

evaporated to dryness and the residual powder or resin placed in a pot still. The pot still has a removable condenser cooled with dry ice. The temperature is gradually raised to 120-170°C. and the pressure reduced to 1-10 microns. Under these conditions, sapogenins sublime and collect on the cooled condenser. Presence or absence of a distillate is the first positive test for sapogenins. Samples which give no distillate are abandoned.

The distillate from step 13 usually has some impurities (yellow pigments and resinous substances). Acetone is an excellent crystallization agent. Sapogenins are insoluble in cold acetone and soluble in the boiling solvent; pigments and resins are soluble in cold acetone. In this way white crystals are secured free from non-sapogenin impurities.

Chromatography

The sapogenins obtained after step 14 usually occur as mixtures. Chromatography has resulted in excellent separations. The sapogenins in benzene solution are adsorbed on alumina andeluted with solvents of increasing polarity. In many cases the separations are sharp and the yields excellent (85-95%).



Tigogenin

The above formula shows the simplest sapogenin type, a saturated monohydroxy sapogenin. Benzene will elute such compounds from alumina. Similar sapogenins which are unsaturated, usually at C_5-C_6, cannot be eluted with benzene but are removed with 2% ether in benzene. Monohydroxy sapogenins which have a carbonyl group are much more tenaciously adsorbed and require elution with chloroform. The presence of an additional hydroxyl, usually at C_2, results in even stronger adsorption. Dihydroxy ketonic sapogenins require 10% ethanol in benzene for elution. Compounds which differ only in spatial arrangement of certain portions of the molecule cannot be separated in this way and require fractional crystallization.

From the foregoing discussion, it is apparent that unknown sapogenins can be classified into at least four groups by their chromatographic behavior. These compounds are now ready for final identification. In all cases acetates are prepared. The melting point data on sapogenins and their acetates will often be almost conclusive. Mixed melting points will be very useful when enough known sapogenin samples are collected. Many of the sapogenins and their acetates form characteristic crystals. Photomicrographs of these compounds will prove helpful when they are again encountered.

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Physical Data

The specific rotation of sapogenins and their acetates is a very useful constant and will often clinch an identification. Unfortunately, many sapogenins were isolated only by Marker. He apparently had an aversion for polarimetry since he gives no specific rotations for the sapogenins he discovered. We do not share this aversion and are determining this constant on all our unknowns. X-ray diffraction patterns and the infrared spectrum of the sapogenins will be very useful, particularly when more known compounds are available. X-ray diffraction gives a very precise "fingerprint" of an unknown. If the patterns of a known and unknown compound coincide they are identical. Shifting even one hydrogen atom gives a totally different diffraction pattern. Hence this technique will prove most useful in distinguishing isomers. Infrared data at present are most useful in rapidly determining the presence or absence of carbonyl groups. It will also give a precise "fingerprint" of the various sapogenins.

In some cases final characterization of a sapogenin may require some structural studies. This is particularly true if a hitherto undiscovered compound is found. Hydrogenation, oxidation and carbonyl reactions are the most commonly used methods. Finally, carbon and hydrogen analyses on sapogenins and their acetates may be necessary. These data are of particular help when dealing with dihydroxy compounds.

Analytical Example

A typical example may show how the foregoing techniques are applied to a plant sample. Yucca gloriosa leaf meal (2.33 kg.) was extracted with alcohol, the extract "defatted" with benzene and then hydrolyzed (step 8). The resultant benzene extract contained 22 grams solids. After alkali treatment (step 10) the solids were reduced to 8.5 grams. Molecular distillation (step 13) resulted in a distillate which weighed 3.0 grams. Crystallization from acetone yielded 1.5 grams purified sapogenin.

Chromatography resulted in the separation of three main fractions; a benzene eluate, 0.316 grams; a chloroform eluate, 0.160 grams; and a 10% ethanol-benzene eluate, 0.613 grams. In addition a total of 0.15 grams were found in various other solvent fractions.

The benzene fraction was recrystallized from methanol and had the following characteristics: Sapogenin m.p. 199-202, $(\alpha)_D^{25}$ pyridine -48; acetate m.p. 202-204, $(\alpha)_D^{25}$ pyridine -54. Of the possible monohydroxy saturated sapogenins, only tigogenin, m.p. 204, $(\alpha)_D^{25}$ pyridine -49, acetate m.p. 202-208, $(\alpha)_D^{25}$ pyridine -57, had constants which were coincident. Hence the unknown is tigogenin.

The chloroform fraction on recrystallization from ether had these properties: m.p. 267-268, acetate m.p. 242-245. Its adsorption behavior classified it as a monohydroxy ketonic sapogenin. Hecogenin, m.p. 268, acetate m.p. 240-250, seemed a logical choice. Fortunately a known sample of hecogenin was available. The fact that the x-ray diffraction patterns of the known and unknown were coincident made the identification certain. Hence this unknown was hecogenin, 12 keto tigogenin.

The 10% ethanol-benzene fraction was recrystallized from ether m.p. 263-263.5, acetate m.p. 236-238. Infrared showed the presence of a carbonyl group. These data most closely fit mannogenin m.p. 264, acetate m.p. 242-243. Mannogenin is 2,3 dihydroxy, 12 keto tigogenin. It will be noted that all three sapogenins isolated from Yucca gloriosa are closely related, i.e. tigogenin, 12 keto

tigogenin, and 2,3 dihydroxy, 12 keto tigogenin.

Final Conclusions

Yucca gloriosa leaves contain 0.65 gm. sapogenin per kg. dry meal or 585 grams per ton (0.065%). Of the sapogenins 56% was mannogenin, 14.5% hecogenin, and 29.5% tigogenin. Mannogenin and hecogenin have a carbonyl group at C_{12} and hence may be potential cortisone precursors.

At the present time 45 samples have been received; this includes division of samples into morphological units. Six samples have been completely studied. It is expected that more rapid results will be obtained since all the experimental details have been worked out.

FLOWSHEET OF METHODS USED FOR ISOLATION AND IDENTIFICATION OF SAPOGENINS

Sample (5-15 kilograms wet weight)

1. Fractionation (leaves, stems, roots, fruits are separated)
2. Coarse Subdivision (slicing, shredding, grinding depending on nature of the sample)
3. Dehydration (60°C. in a tray drier)
4. Grinding (4-5 mm. particle size)

Dry meal (1-5 kg.)

5. Alcohol Extraction (1. Hot 70% ethanol)
   (2. Hot 95% ethanol)

Alcohol Extract (saponins) — Residual meal (discard)

6. Concentrate (final volume 1-2 liters)
7. Liquid-Liquid Extraction with Benzene (4-5 hours)

Aqueous Alcohol Extract (saponins) — Benzene Extract (fats, sterols etc., discard)

8. Hydrolysis with 2N-HCl at 65°C. + Benzene Extraction

Benzene Extract (1-10% sapogenin) — Aqueous alcohol (discard)

9. Concentrate
10. Reflux with Methanol-KOH (15 minutes)
11. Extract with Benzene

Benzene Extract (10-20% sapogenin) — Methanol-KOH containing phenolic compounds and resin acids (discard)

12. Concentrate to dryness

13. Molecular Distillation (120-170°C, 1-10 microns)

- Distillate (25-75% sapogenin)
- Residue (resins, discard)

14. Acetone Crystallization

- Acetone Insoluble Crystals (100% sapogenin)
- Acetone Soluble (resins, pigments, gums, discard)

15. Chromatography on Merck Alumina (adsorb from benzene solution)

16. Elute with

- Benzene → Monohydroxy, saturated, non-ketonic
- 2% ether in Benzene → Monohydroxy, unsaturated, non-ketonic
- Chloroform → Monohydroxy, ketonic
- 10% ethanol in Benzene → Dihydroxy, ketonic

17. Prepare sapogenin acetates

18. Determine melting points and crystalline structure of sapogenins and sapogenin acetates (Microscope Hotstage equipped with polarizing devices)

19. Determination of specific rotation of sapogenins and their acetates

20. Chemical characterization (hydrogenation, oxidation, and carbonyl reactions)

21. X-ray diffraction pattern of sapogenins

22. Infrared spectra of sapogenins

23. C, H, determinations of sapogenins and their acetates.